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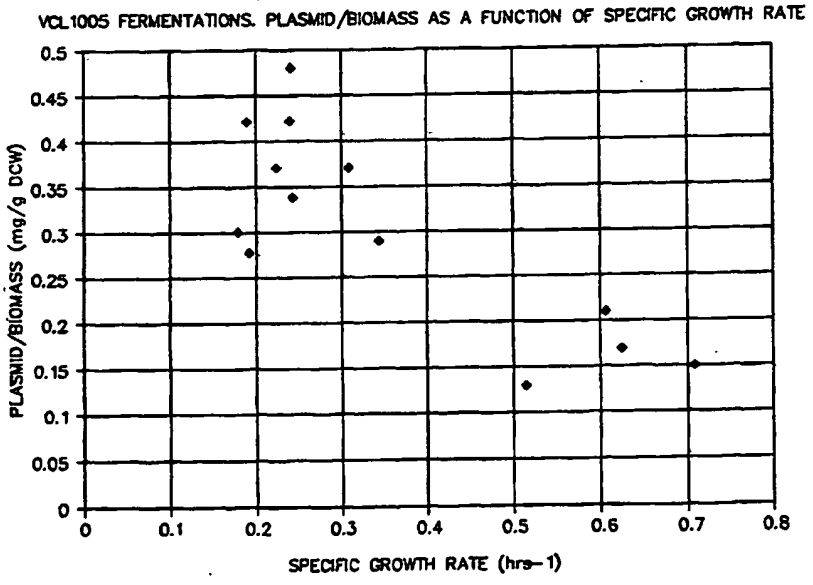

(57) Abstract

A method for producing production scale quantities of pharmaceutical grade plasmid DNA in a microorganism at high efficiencies, comprising the steps of providing a microorganism culture, wherein the microorganisms include a recombinant plasmid, growing the microorganisms in culture under conditions that limit growth of the microorganisms during exponential phase to a growth rate of no more than about 0.35/hr, and then purifying the plasmid DNA from the microorganisms.

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OPTIMIZED HIGH-YIELD PRODUCTION OF PLASMID DNA

FIELD OF THE INVENTION

This invention relates to a fermentation process to produce milligram and gram quantities of plasmid DNA from bacteria, mammalian cells, and yeast. The method is concerned with the manufacturing process for the production of plasmid DNA for use as a pharmaceutical, as in gene therapy products.

BACKGROUND OF THE INVENTION

Direct injection of plasmid DNA is an attractive alternative to viral-based gene delivery systems. Several animal studies have demonstrated the feasibility of direct injection of plasmid DNA for gene therapy (Acsadi et al., 1991, Direct gene transfer and expression into rate heart *in vivo*. New Biol. 3, pp. 71-81; Buttrick et al., 1992, Behavior of genes directly injected into rate heart *in vivo*. Circ. Res. 70, pp 193-198.; Leinwald et al., 1991, Gene transfer into cardiac myocytes *in vivo*. Trends Cardiovasc. Med. 1, pp. 271-276.; Nabel et al., 1989, Recombinant gene expression *in vivo* within endothelial cells of the arterial wall. Science. 244, pp. 1342-1344., 1990, Site-specific gene expression *in vivo* by direct gene transfer into the arterial wall. Science. 249, pp 1285-1288.; 1992b, Gene transfer in vivo with DNA-liposome complexes: Lack of autoimmunity and gonadal localization. Hum. Gene Ther. 3, pp. 649-656., 1992c Transduction of a foreign histocompatibility gene into the arterial wall induces vasculitis. Proc. Natl. Acad. Sci. USA 89, pp. 5157-5161.; Stewart et al., 1992, Gene transfer in vivo with DNA-liposome complexes: Safety and acute toxicity in mice. Hum. Gene Ther. 3, pp. 265-267.) and this approach has proceeded to human clinical investigations (Caplen et al., 1994, Gene therapy for cystic fibrosis in human by liposome-mediated DNA transfer: The production of resources and the regulatory process. Gene Ther. 1, pp 139-147.; Nabel et al., 1992a, Clinical Protocol: Immunotherapy of malignancy by *in vivo* gene transfer into tumors. Hum. Gene Ther. 3, pp. 399-410., 1993, Direct gene transfer with DNA-liposome complexes in melanoma: Expression, biologic activity, and lack of toxicity in humans. Proc. Natl. Acad. Sci. USA 90, pp. 11307-11311). Human clinical trials of direct *in vivo* transfer gene therapy require the development of scaleable manufacturing processes that reproducibly meet the quality criteria of purity, potency, efficacy, and safety for a recombinant drug substance. (Horn et al., 1995, Purification of Pharmaceutical-Grade Plasmid DNA for Human Clinical Trials. Human Gene Therapy). There is a need to develop a high-yielding fermentation for the production of plasmid DNA for human clinical trials that is 1) capable of producing plasmid yields higher than those achieved by current methods; 2) comprehensive in that it can be used for the production of a variety of constructs; 3) reproducible; and, 4) compliant with current regulatory standards.

Plasmid DNA production has historically been a technique used to support general laboratory research. Conventional, state-of-the-art methods of plasmid DNA production are based on the shake flask method under uncontrolled conditions involving: 1) growth in rich media such as Terrific Broth (Sambrook, et al., 1992, Molecular Cloning: A Laboratory Manual (2nd ed.), Cold Spring Harbor, NY., (Cold Spring Harbor

Laboratory Press)) and Circle Grow (Bio 101, La Jolla, Ca.); 2) addition of chloramphenicol to the medium, which alters protein synthesis; or 3) alteration of the genotype of the host strain (relA mutation) (Herman, et al., 1994, Regulation of Replication of Plasmid pBR322 in Amino Acid-Starved *Escherichia coli* Strains. Mol Gen Genet, Vol. 243, p. 374-378.). Plasmid DNA destined to be used in a pharmaceutical application requires scaleable and well-controlled manufacturing methods. Laboratory methods such as these are not suitable for large scale manufacturing especially in a GMP environment.

Current batch methods are inefficient in both overall plasmid yield and in converting raw materials to product. The concentration of substrate in yeast extract and peptones is usually very high, and these materials may also be expensive. When grown under batch conditions, the culture is susceptible to the repressive effects of rapidly utilized carbon sources. Such repressive effects result in lower plasmid yields and higher yields of metabolic by-products such as acetate and carbon dioxide (Ward, et al., 1989, Fermentation Biotechnology. Inglewood Cliffs, New Jersey (Prentice Hall Publishers); Luli et al., 1990, Comparison of Growth, Acetate Production, and Acetate Production and Acetate Inhibition of *E. coli* Strains in Batch and Fed-Batch Cultures. Applied and Environmental Microbiology, Vol. 56, No. 4, p. 1004-1011.) As a result of these repressive effects, the culture enters stationary phase prematurely, thereby reducing plasmid DNA yield. Thus, producing growth-associated products like plasmid DNA in batch-type fermentations is inefficient because product formation only occurs during a fraction of each fermentation cycle.

The fed-batch process described here results in higher overall plasmid DNA yields than the batch process by extending the exponential phase of the fermentation. This process application has major implications in terms of final product cost and successful scale-up.

SUMMARY OF THE INVENTION

We have developed a fed-batch fermentation process for the production of plasmid DNA that: 1) identifies the relationship between cellular specific growth rate and plasmid accumulation rate during the exponential phase of growth (growth-associated product); 2) increases overall plasmid yield by extending the exponential growth phase of the culture; 3) does not require alteration of the genotype of the host strain or the plasmid; 4) does not require supplementation of the medium with chloramphenicol; 5) is comprehensive in that it can be used to produce different recombinant *E. coli* plasmids; 6) has a higher efficiency of raw material conversion to product than the batch fermentation method.

The invention provides a process for producing plasmid DNA, comprising the steps of: (a) providing a microorganism culture wherein the microorganisms contain recombinant plasmids; (b) controlling the growth conditions of the culture to limit the rate of growth during the exponential phase to about 0.35/hour or less; and (c) purifying the plasmid DNA from the microorganisms.

According to the invention, the growing step (b) may also include measuring the growth rate of the microorganisms in the culture.

Also, according to the invention, the growth rate may be maintained by feeding one or more

nutrients to the microorganisms in quantities increasing at a rate approximately equal to the growth rate.

In one embodiment of the invention, the feeding of the culture is done periodically. In another embodiment of the invention, the feeding of the culture is done continuously.

Where the growth rate is controlled by feeding one or more nutrients to the culture at a controlled rate, the nutrient may be a carbon source. In one embodiment of the invention, the carbon source may be either glucose, glycerol, sucrose, maltose, fructose, lactose, corn syrup, molasses, honey, or a combination thereof. In a preferred embodiment, the carbon source is glucose.

In another embodiment of the invention, the growth rate may be controlled by the feeding of an organic molecule for which the microorganisms in the culture are auxotrophic. The organic molecule may be an amino acid, e.g. L-leucine.

In another embodiment of the invention, the growth rate may be limited by the controlled feeding of an inorganic substance required by the microorganisms for optimal growth.

One aspect of the invention uses eukaryotic microorganisms for the culture. In a preferred embodiment of this aspect of the invention, the eukaryotic microorganism is yeast.

Another aspect of the invention uses prokaryotic microorganisms for the culture. In the most preferred embodiment of the invention, the microorganisms of the culture are *E. coli*.

The invention is further embodied by a purifying step that results in pharmaceutical grade DNA. In a preferred embodiment of the invention, the DNA yield may be about 160 mg per 10ℓ batch. The yield efficiency of plasmid DNA may be about 0.04%, where yield efficiency is defined as the mass of plasmid DNA produced, divided by the mass of carbon source used to feed the culture.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a graph showing the growth-associated relationship between plasmid yield per unit of biomass and specific growth rate.

DETAILED DESCRIPTION OF THE INVENTION

Current state of the art techniques to increase the production of plasmid DNA via fermentation are based on 1) the batch fermentation process which has a limited exponential growth phase; 2) production media such as Terrific Broth (Sambrook et al., 1992 Molecular Cloning: A Laboratory Manual (2nd ed.), Cold Spring Harbor, NY., (Cold Spring Harbor Laboratory Press) and Circle Grow (BIO 101, La Jolla); 3) adding chloramphenicol to the medium which alters protein synthesis and enhances plasmid DNA replication; and, 4) altering the genotype of the host strain (relA mutation) (Herman et al., 1994 Regulation of Replication of Plasmid pBR322 in Amino Acid-Starved *Escherichia coli* Strains. Mol Gen Genet (1994), Vol. 243, p. 374-378.).

We have developed a fed-batch fermentation process for the production of plasmid DNA that: 1) identifies the relationship between cellular specific growth rate and plasmid accumulation rate during the

exponential phase of growth (growth-associated product); 2) increases overall plasmid yield by extending the exponential growth phase of the culture; 3) does not require alteration of the genotype of the host strain or the plasmid; 4) does not require supplementation of the medium with chloramphenicol; 5) is comprehensive in that it can be used to produce different recombinant *E. coli* plasmids; 6) has a higher efficiency of raw material conversion to product than the batch fermentation method.

The invention provides a process for producing plasmid DNA, comprising the steps of: (a) providing a microorganism culture wherein the microorganisms contain recombinant plasmids; (b) controlling the growth conditions of the culture to limit the rate of growth during the exponential phase to about 0.35/hour or less; and (c) purifying the plasmid DNA from the microorganisms.

MICROORGANISMS

The microorganisms of the invention include all microorganisms capable of stably maintaining and replicating plasmids. The optimal host for a given plasmid may vary, and this invention contemplates the selection of a microorganism host best suited for scale-up production of the desired plasmid.

The most common host for production of recombinant plasmids is *E. coli*. A most preferred embodiment of the invention employs *E. coli* as the host organism for plasmid production. There are numerous strains of *E. coli* in use for various applications, and one of ordinary skill in the art would be capable of selecting an appropriate strain of *E. coli* for the optimization of plasmid production.

Any other bacterium capable of utilization as a host for plasmid production is included in this invention. Because of certain differences among bacterial species in their replication or modification of plasmid DNA, in some situations the optimal bacterial host for production of a particular recombinant plasmid will be a non-*E. coli* bacterium. Non-limiting examples of non-*E. coli* bacteria that may be employed in the present invention are *Pseudomonas*, *Bacillus*, and *Xanthomonas*.

A person of ordinary skill in the art would be able to select an appropriate host and apply this invention to the particular plasmid DNA production scenario.

In addition to bacteria, other microorganisms can also harbor and stably replicate plasmids. The most common eukaryotic cell used in procedures involving plasmids is yeast. The various different species and strains of yeast that may be used are all contemplated in this invention. Specifically, *Saccharomyces cereviseae* and *Schizosaccharomyces pombe* constitute preferred yeast species. *Hansenula*, *Pichia*, and *Candida* are other non-limiting examples of yeast species or strains that may be used according to the invention. Other species and strains are known in the art, and one of ordinary skill would be able to make an appropriate selection of a workable host for optimized plasmid production according to this invention.

Other non-yeast eukaryotes, such as mammalian cells and *Aspergillus* and Actinomycete fungi, known in the art to stably maintain and replicate recombinant plasmids, are also embodied as hosts for optimized plasmid production according to this invention.

PLASMIDS

This invention relates to optimized production of recombinant plasmids. A recombinant plasmid is a circular DNA molecule containing DNA sequences that have been recombined from other DNA sequences, whether those sequences were newly created or previously existing, or whether some sequences were newly created and others were previously existing.

The DNA sequences subject to recombination can include linkers, whole genes, chimeric genes, gene fragments, products of random or directed mutagenesis, promoters, enhancers, silencers, terminators, exons, introns, transposable elements, random or specific spacers, and any other DNA sequence found in, or inserted into, a plasmid.

Under certain conditions, plasmid DNA can exist in a linear or concatameric form. All DNA sequences that are plasmids or are derived from plasmids in whatever configuration, are contemplated as embodiments of this invention.

Plasmids may sometimes be classified based on their derivation (e.g., a pUC derivative plasmid); on the origin of particular sequences they contain (e.g., a chick lysozyme plasmid); on relative copy number in the host (e.g., a high- or low-copy number plasmid); on antibiotic resistance they confer (e.g., a kanamycin resistant plasmid); on their preferred host (e.g., a yeast plasmid); or on other bases useful for organizing and classifying the great diversity of recombinant plasmids. Such well known plasmids as pBR322, pUC19, V1J, pUC18, and the IncFII (Runaway) class of inducible plasmids, are a few of the many examples of plasmids suitable for use in the present invention.

According to the invention, all recombinant plasmids are contemplated, regardless of how they are classified. A person of ordinary skill in the art would be able to apply the process of this invention to any recombinant plasmid without undue experimentation. The use of certain plasmids in the examples of this invention are only for demonstration purposes are do not limit the scope of the applicability of the invention.

CONTROL OF CONDITIONS TO LIMIT GROWTH RATE

According to the invention, the growth rate of the culture is limited so as to achieve an optimal yield of plasmid DNA. In one embodiment of the invention, the limitation of the growth rate is achieved by controlled feeding of one or more nutrients required by the culture for growth. In alternative embodiments, limitation of growth rate is achieved by controlling such factors as oxygen supply to the culture, incubation temperature, and culture pH.

Nutrients

In one embodiment of the invention, the limitation of the growth rate is achieved by controlled feeding of one or more nutrients required by the culture for growth. A preferred embodiment of the invention regulates culture growth rate by controlled delivery of the primary carbon source. In alternative embodiments, growth rate of the culture is limited by controlled delivery of other organic molecules for which the microorganisms of the culture are auxotrophic, or by the controlled delivery of inorganic substances necessary

for growth of the microorganisms of the culture.

In a preferred embodiment, the controlled nutrient is the primary carbon source of the growing culture. A most preferred embodiment of the invention uses glucose as the primary carbon source subject to controlled delivery. Other commonly-used carbon sources include glycerol, sucrose, maltose, fructose, lactose, corn syrup, molasses, and honey. Additional carbon sources are also contemplated. As a non-limiting example, yeast extract provides a variety of nutrients to a culture, and in some media serves as the exclusive carbon source as well. Accordingly, a fed-batch fermentation, wherein the controlled feeding is of the nutrient mix, is contemplated as an embodiment of the invention. Feeding can be accomplished continuously or periodically.

The growth of the culture can also be limited to achieve an optimal rate by controlled delivery of other organic molecules for which the microorganisms of the culture are auxotrophic. Any auxotrophic microorganism will fail to grow if the molecule for which it is auxotrophic is not available in the culture medium. Accordingly, the growth rate of a culture can be limited by restricting the availability of an essential organic component. One embodiment of this aspect of the invention employs amino acids as the limiting organic molecules. In a preferred embodiment, the limiting amino acid is L-leucine. All other amino acids are also contemplated as possible limiting nutrients according to the invention.

The invention contemplates the use of any auxotrophic strains of microorganisms capable of harboring and stably replicating a recombinant plasmid. When such an auxotrophic strain is used, the growth rate of the culture can thus be regulated by the supply of the limiting nutrient. Examples of other organic molecules for which particular microorganism strains may be auxotrophic include, but are not limited to: nucleotides, fatty acids, vitamins, growth factors, and coenzymes. Accordingly, any organic molecule whose controlled delivery could limit the growth rate of a microorganism culture is contemplated in this invention. A person of ordinary skill in the art could determine an appropriate combination of auxotrophic strain and organic molecule to practice the process of the invention.

In addition to the primary carbon source and other organic molecules of the culture medium, a variety of inorganic components necessary for proper growth of a microorganism may be used with the aim of limiting growth rate by controlling delivery of the component to the culture. The growth rate of certain microorganisms will depend on the available concentration of inorganic vitamins, growth factors, coenzymes, and ions such as phosphates, magnesium, and sulfates. These are given as examples and not as a complete list of possible components of a nutrient medium that may have the desired effect. Any culture nutrient, organic or inorganic, whose presence or absence can limit or attenuate the growth rate of a culture of microorganisms, is contemplated by this invention. A person of ordinary skill in the art would be able to practice the invention according to this embodiment without undue experimentation, but simply by knowing the components of a given nutrient solution empirically determining which nutrients, when supplied in limiting quantities, attenuate the growth rate of the selected microorganism.

In any of the previously discussed embodiments of this aspect of the invention, the controlled

delivery of a chosen nutrient or combination of nutrients to achieve the desired rate of growth can be accomplished by delivering a selected quantity of the nutrient periodically at set intervals. In an alternative embodiment, the nutrient or nutrients may be delivered continuously at a pre-programmed rate to match or otherwise complement the desired optimal growth rate. In another embodiment of this aspect of the invention, the delivery of a nutrient may be controlled and continuously adjusted to match actual detected growth rates of the culture in real time.

Other Means for Controlling Growth Rate

Beyond regulation of the delivery of nutrients, other characteristics of the culture environment may also have the desired capability of limiting the growth rate to the optimal level for efficient production of plasmid DNA. In one embodiment of the invention, the desired optimal growth rate is achieved by regulation of the incubation temperature. In a preferred embodiment, the incubation temperature of a culture of *E. coli* is kept at 30 °C, rather than at 37 °C, the temperature to which *E. coli* is environmentally best adapted. In alternative embodiments, a culture could be maintained within a temperature range from 26 to 29 °C, or less preferably within a temperature range from 22 to 25 °C, in order to lower the growth rate to a desired rate for optimal yield of plasmid DNA. Certain bacterial cultures may achieve the desired rate of growth be maintenance between the temperatures of 31 and 33 °C, or even between the temperatures of 34 and 36 °C. Nothing in this discussion is intended to limit the practice of the invention to below-normal temperatures only. Any temperature at which a desired growth rate is achieved is contemplated in the present invention.

Meanwhile, a culture of yeast, well adapted to grow at 30 °C, may be grown at a different temperature so as to achieve the desired rate of growth according to the invention. A person skilled in the art would easily be able to empirically determine the temperature that is best suited for any particular combination of nutrients, microorganism, and plasmid.

The growth rates of both aerobic and anaerobic microorganisms can be affected by the supply of oxygen in the growth medium. Controlled delivery of oxygen to achieve the desired growth rate is contemplated as an embodiment of the present invention. This can be accomplished in a fermentor by programming the actual delivery of oxygen into a mixed-gas feed to the culture. Alternatively, the attenuated presence of an oxygen scavenger in the fermentor environment may also effectively regulate growth rate by controlling the amount of oxygen available to a culture of microorganisms. In a preferred embodiment, oxygen supply is detected in the culture and is adjusted by alteration of the agitation or mixing rate of the vessel. Other means of regulating growth rates by oxygen availability are also contemplated in the present invention.

Many microorganisms have a preferred culture pH for maximal growth. Accordingly, growth rates can be limited by periodic or continuous adjustment of the pH of the culture medium during fermentation. In one embodiment of the invention, this is accomplished by computer- or technician-controlled responsive delivery of acid or base into the culture medium to adjust for fluctuations in pH.

In any of the previously discussed embodiments of this aspect of the invention, the attenuation of a chosen environmental factor or set of environmental factors so as to achieve the desired optimal growth

rate, can be accomplished by adjusting the environmental conditions of the culture periodically at set intervals. In an alternative embodiment, the environmental factor or factors may be adjusted continuously at a pre-programmed rate to match or otherwise complement the desired optimal growth rate. In another embodiment of this aspect of the invention, nature of a given environmental factor or factors may be controlled and continuously adjusted to match actual detected growth rates of the culture in real time.

MEASURING GROWTH AND GROWTH RATE

This invention relates to the inverse association between efficient intracellular plasmid accumulation and the rate of growth of the host culture of microorganisms. As discussed above, the invention contemplates all ways in which the growth rate of a culture of microorganisms can be regulated, so as to optimize overall yield and efficiency of plasmid production. However, in order to know whether and how much to attenuate the growth rate by employing one or more of the rate control means, one must know the actual growth rate of the culture.

One embodiment of the invention is to determine growth rate by periodic sampling or other measurement of the culture and comparison in the change of accumulated biomass or other relevant measure over the sampling interval. In an alternative embodiment, sampling is done continuously, and a current growth rate is constantly indicated.

The invention contemplates various sampling methods for determining growth rate, including, but not limited to, measurements of: culture turbidity, electrical conductivity of the culture medium, direct cell count, direct biomass measurement, depletion of a component of the culture medium, and accumulation of a by-product or waste product of the culture.

In a preferred embodiment of the invention, an on-line optical density probe capable of spectrophotometric analysis is used to determine the turbidity of the culture. A change in turbidity per unit time is directly proportional to the increase in number of cells in the culture, and the growth rate can be directly calculated therefrom.

In an alternative embodiment, predetermined volumes of culture will pass through an automated cell counter either continuously or at predetermined intervals. Increase of cell count over time will give a direct indication of culture growth rate.

In another embodiment of the invention, a sample is withdrawn from the culture and subjected to centrifugation at accelerations sufficient to efficiently separate cells from liquid medium. After aspiration of the supernatant medium, the sample tube is weighed and compared with a tare weight to directly determine biomass. A measured change in biomass over time will give a direct indication of the growth of the culture.

An alternative embodiment of the invention involves sampling an easily assayed physiological characteristic of the microorganism, such as the respiratory coefficient (RQ = CO_2/O_2), that would be likely to change as growth continues. The magnitude of the change in such a quantitative physiological indicator

should be proportional to the rate of growth of the microorganism culture.

Other means for measuring growth are known in the art, and are contemplated in the present invention. A person skilled in the art could easily apply one of these other means for measuring growth rate to the practice of the present invention.

OPTIMAL RATE

This invention relates to the inverse association between efficient plasmid production and growth rate. In conditions of rapid growth and cell division in a culture of microorganisms that contain plasmids, the overall yield and efficiency of plasmid production is relatively low. However, in conditions of relatively lower growth rate by the cultured cells, there is an inversely corresponding increase in the overall yield and efficiency of plasmid production.

According to the invention, there is an optimal rate of growth of host cells in culture, at which the overall yield and efficiency of plasmid production is optimized. The optimal rate will occur within a range of useful efficiency, consisting of at least an upper limit and more usually an upper limit and a lower limit. The upper limit is dictated by efficiency concerns: the point at which overall yield and efficiency of plasmid production appreciably diminish is the point defining the upper limit. The lower limit is dictated by practical considerations such as the need to grow enough cells, during a fermentation period of finite duration, to have a useful gross quantity of plasmid product at the end of the fermentation period.

In one embodiment of the invention, the optimal growth rate can be defined as less than about 0.35/hour. Specific Growth Rate (μ) is defined and determined by the following formula:

$$\mu = \frac{\ell n(N/N_0)}{\Delta t}$$

where ℓn is the natural logarithm, N is a measure of the number of cells in the culture at a given time, N_0 is a measure of the number of cells in the culture at an earlier time, and Δt is the change in time between the determinations of N and N_0. In one embodiment, N is OD_{600}, the spectrophotometric absorbance (optical density) of the culture measured at a light wavelength of 600 nm, and measured at time t. N_0 is OD_{600} measured at time at t-n. Δt and n are equivalent terms that both equal the elapsed time between the time of measurement N and the time of measurement N_0. The formula may be applied to any embodiment of the invention capable of quantitatively expressing the cell number, or a value proportional thereto, and need not be limited to spectrophotometric quantitations only.

Other embodiments of the invention define ranges of useful efficiency by identifying preferred upper and lower limits. As upper limits, about 0.35/hour is the most preferable embodiment, with preferable embodiments having upper limits of about 0.30/hour or about 0.25/hour. Less preferable embodiments may have upper limits as low as about 0.20/hour or about 0.15/hour.

A preferred embodiment of the invention has a lower limit of about 0.01/hour, with more preferable

embodiments having as lower limits about 0.05/hour or about 0.10/hour. Lower limits contemplated by the invention may be as high as about 0.15/hour or about 0.20/hour.

Although the embodiments of the invention clearly prefer an upper limit of about 0.35/hour, workable but inferior results may be achieved with upper limits of about 0.40/hour, about 0.45/hour, and even about 0.50/hour, and in some circumstances, these limits should be considered to be part of the present invention.

The optimal growth rates, and corresponding feed rates, as discussed above, can easily be empirically determined, or can be determined iteratively with a short series of feeding, growth, and yield trials that will not require undue experimentation for a person of skill in the art to practice the process of the present invention.

PURIFICATION OF PLASMID DNA

The invention is further embodied by a purifying step that results in pharmaceutical grade DNA. In a preferred embodiment of the invention, the DNA yield may be at least about 160 mg per 10ℓ batch. Other preferable embodiments of the invention have DNA yields of about 140 mg per 10ℓ batch and about 120 mg per 10ℓ batch. A less preferred embodiment contemplated by the invention gives a yield of about 80 mg per 10ℓ batch. By practicing the present invention, a person skilled in the art could easily optimize production of plasmid DNA. Because the actual yield will depend on a variety of factors controlled and contemplated by this invention, whatever the actual yield in a given circumstance is, if optimized according to the invention, the optimized yield is a contemplated embodiment of the present invention.

One preferred method of purification of the plasmid is described in detail in our copending patent application, "Production of Pharmaceutical Grade Plasmid DNA," Serial No. 08/192,151, incorporated herein by reference.

Additional preferred embodiments of the purification step of this invention employ other methods of plasmid DNA purification known in the art and widely practiced. There are two widely used laboratory methods for the preparation of a crude lysate enriched with plasmid DNA: the boiling method and the alkaline lysis method. Both commonly utilize chicken egg-white lysozyme to break up the bacterial cell wall. Laboratory scale centrifugation is often implemented to separate cellular debris from the crude lysate. Pancreatic RNase is frequently employed to reduce host-derived RNA. Organic extraction with phenol:chloroform:isoamyl alcohol or a variation of this mixture is typically used to reduce contaminating proteins.

The plasmid DNA isolated from the crude lysate is further purified by cesium chloride/ethidium bromide (CsCl/EtBr) equilibrium ultra-centrifugation. Due to density differences created by the different binding capacities of EtBr to covalently closed circular plasmid DNA, RNA and chromosomal DNA, these three different nucleic acids can be resolved into enriched fractions by CsCl gradient ultra-centrifugation.

There are variations of the methodology described above where the crude lysate is treated with pancreatic RNase followed by an alkali/detergent treatment to reduce chromosomal DNA. An organic

extraction with phenol:chloroform is followed by precipitation of DNA by ethanol, re-suspension and a polyethylene glycol (PEG) precipitation of DNA.

EFFICIENCY OF CONVERSION FROM CARBON SOURCE TO PLASMID DNA

This invention is related to efficient large-scale production of plasmid DNA. The efficiencies achieved are due to an understanding and a manipulation of the relationship between growth rate of the microorganisms in the culture, and the accumulation rate of plasmids within the microorganisms. A most preferred embodiment of this aspect of the invention gives a plasmid yield efficiency of about 0.04%. The term "plasmid yield efficiency" is defined as the total mass of plasmid DNA produced, as a percentage of the total mass of the primary carbon source used to feed the culture. Another preferred embodiment of this invention gives a plasmid yield efficiency of about 0.035%. Less preferred embodiments of the invention give plasmid yield efficiencies of 0.03% and 0.025%. A person skilled in the art, practicing the present invention, would be able to achieve these yield efficiencies without undue experimentation.

In some cases, plasmid yield efficiencies according to the present invention may be as low as 0.02% or even 0.015%. Where these efficiencies represent an improved or optimized efficiency in conversion of supplied carbon source to the plasmid, from practice of this invention, such efficiencies are contemplated by this invention.

EXPERIMENTAL RESULTS

Plasmid Accumulation in Scale-Up Batches has an Inverse Relationship to Culture Growth Rate

Plasmid DNA was produced initially in shake flasks with Terrific Broth (Sambrook, et al., 1992, Molecular Cloning: A Laboratory Manual (2nd ed.), Cold Spring Harbor, NY., (Cold Spring Harbor Laboratory Press)). Subsequently the Terrific Broth batch fermentation was scaled-up to a fermentor. Because the Terrific Broth method was designed for shake flasks and no modifications were made to the method before transferring it to the fermentor, plasmid DNA yield was not increased.

In order to modify the Terrific Broth fermentation to make it more suitable for large scale process fermentation, glycerol, the primary carbon source in the medium, was increased. The purpose of this change was to increase the biomass by providing more carbon to the culture. It was assumed that where there was a greater production of biomass, there would also be a greater overall amount of plasmid DNA produced.

However, initial attempts to modify the Terrific Broth batch fermentation process and increase plasmid DNA yield from batch fermentations resulted in increases in biomass but no significant increases in plasmid yield. For example, as shown in Table 1, below, in producing plasmid VCL1102 a five-fold increase in glycerol resulted in a three-fold increase in biomass, but gave only a slight increase in overall plasmid yield. Contrary to our expectation that increasing the glycerol in the broth would increase biomass and plasmid yield, the ratio of plasmid to biomass decreased. The plasmid DNA levels reached a plateau while biomass continued to increase, resulting in a lower amount of product for the same mass of cells. This effectively

lowered the "purity" of the product in the culture, adversely affecting downstream processing requiring larger and more costly equipment to purify the same amount of product.

TABLE 1.

Effects of Increased Carbon Source on Overall Plasmid Yield

Conditions	Plasmid	Biomass (g)	Plasmid Yield (mg/ℓ)
1X Glycerol	VCL1005	356	2.7
5X Glycerol	VCL1005	718	8.9
1X Glycerol	VCL1102	382	3.1
5X Glycerol	VCL1102	1169	3.9

This disproportional relationship led to the conclusion that plasmid DNA is a growth-associated product. Many cell products are growth associated. The term "growth associated" simply means that the production or accumulation of a particular product is associated with the growth rate of the culture. Some products have a directly proportional growth association, in which case the higher the growth rate of the culture, the greater the accumulation of the product.

Other products have an inverse relationship to growth rate. This means that higher growth rates of the culture give diminished efficiency of product accumulation. For an inversely growth-related product, it would therefore be important to control growth rates at or below a critical level, allowing the optimal efficiency of accumulation of the product.

In the case of plasmid DNA, our data indicate that the growth association is inverse. That is, plasmid accumulates intracellularly less efficiently at higher growth rates than it does at lower growth rates. Figure 1 shows that cultures with growth rates less than 0.35/hour consistently have a higher plasmid/biomass ratio than cultures with growth rates greater than 0.5/hour.

Development of an Optimal Fed-Batch Fermentation Process

Because of the relationship between growth rate and plasmid DNA accumulation, it is necessary to employ a fermentation process that extends the phase during which plasmid DNA is produced. Extension of this growth phase of the culture can be accomplished by means of a fed-batch fermentation where a nutrient, usually the carbon source, is fed at a rate which controls the rate at which the culture grows (Specific Growth Rate, μ, as defined in the formula above).

It is for this reason that we adapted fed-batch fermentation techniques to create a novel process that is suitable to manufacture plasmid DNA for such uses as gene therapy. The fed-batch fermentation process described here is accomplished by preparing a medium where the nutrients are defined, with the exception of yeast extract. The carbon source, in the form of dextrose, is fed exponentially and is under

computer control. Dextrose was chosen to replace glycerol as the carbon source because it is converted more efficiently to product and biomass by the culture and is more suitable in a fed-batch application. Other nutrients such as nitrogen, magnesium, vitamins, and trace elements are fed along with the dextrose to increase the efficiency at which they are converted to biomass.

Advantages of the Invention

A. Overall Yield

This fed-batch fermentation process is capable of producing various plasmids in milligram and gram quantities. As an example, refer to Table 2 below.

TABLE 2.

Plasmid Yield Comparison of Different Constructs Under Batch and Fed-Batch Conditions

	VCL1005		VCL1102	
	Batch	Fed-Batch	Batch	Fed-Batch
Plasmid Yield (mg/ℓ)	3.4	16	3.0	18
Dry Cell Weight	9.9	45	6.6	50
Plasmid/DCW (mg/g)	0.35	0.36	0.45	0.36
Plasmid (mg/10ℓ batch)	34	160	30	180

As Table 2 shows, the fed-batch method results in much greater overall plasmid yields per 10ℓ batch. Although the plasmid to dry cell weight ratio stays about the same, the overall yield is much greater because the exponential phase of growth in the cultures can be significantly prolonged. This results in much greater biomass production per batch.

The capability to increase plasmid DNA concentrations does not rely on any genetic manipulation of the host chromosome or of the plasmid and does not introduce chemicals into the medium that cause repression of protein synthesis such as chloramphenicol. The increases in plasmid yield result from growing the culture at a specified growth rate (as determined by the feed rate of the carbon source, or of any other vital nutrient) and extending the phase in which plasmid DNA is produced. These attributes clearly distinguish the fermentation process described here from current state-of-the-art methods and make it especially well suited for the manufacture of plasmid DNA used in products for gene therapy.

B. Efficiency

The conversion of primary carbon source to plasmid DNA is the measure of efficiency of the fed-batch process. The increased biomass generated in the fed-batch process would be of no value if the efficiency of plasmid production were not increased or at least preserved in comparison with the state-of-the-art batch fermentation process. As an example, see Table 3 below.

TABLE 3.

Plasmid Yield Efficiency Comparison of Batch and Fed-Batch Processes

Conditions	Total Carbon (g/ℓ)	Plasmid Yield (mg/ℓ)	% Carbon to Plasmid
1X Glycerol Batch	14.6	3.0	0.020
5X Glycerol Batch	21.0	3.9	0.018
Fed-Batch	44.0	18.0	0.041

The optimization process described herein has the property of doubling the efficiency of conversion of carbon source to plasmid DNA as demonstrated above. Note that attempted scale-up by a five-fold increase in the carbon source results in a decreased plasmid yield efficiency from 0.020% to 0.018%. In contrast, scale-up by the fed-batch method of this invention resulted in a plasmid yield efficiency of 0.041% -- a doubling of the efficiency.

EXAMPLE 1. Medium Development

Eight liters of Base Medium, as described below, was inoculated with a culture of *E. coli* containing plasmid VCL1005. Conditions of fermentation were maintained at 30°C, 20% O_2, and pH 7.0 throughout the experiment. At t = 3.5 hours, controlled delivery of the Feed Medium, as described below, was begun. From times t= 3.5 hours to t = 6.5 hours, Feed Medium was delivered such that the rate of addition of glucose to the culture was 1.5 g/ℓhr. Over the next seven hours, glucose was added at an escalating rate going from 1.5 g/ℓhr to 3.0 g/ℓhr. To provide for exponential growth of the culture, the delivery rate of glucose was increased exponentially over the remainder of the fermentation period.

Spectrophotometric measurements of the turbidity of the culture were taken periodically to record growth rates. When the culture entered the late logarithmic phase of growth, nutrients from the group of Supplemental Nutrients, as described below, were added to the culture. By adding each nutrient separately and continuing to chart the growth rate it was determined that, even under fed-batch conditions, supplemental L-leucine must be added to sustain the logarithmic growth of the culture.

<u>Base Medium</u>

Glucose	1.0 g/ℓ (+/- 1 g/ℓ)
Ammonium sulfate	5.0 g/ℓ (+/- 2 g/ℓ)
Yeast extract	5.0 g/ℓ (+/- 2 g/ℓ)
Potassium phosphate, dibasic	7.0 g/ℓ (+/- 2 g/ℓ)
Potassium phosphate, monobasic	8.0 g/ℓ (+/- 2 g/ℓ)
Magnesium sulfate	1.0 g/ℓ (+/- 1 g/ℓ)
Trace elements	1.0 ml/ℓ (+/- 1 ml/ℓ)
Vitamins	1.0 ml/ℓ (+/- 1 ml/ℓ)
Kanamycin	50 mg/ℓ (+/- 10 mg/ℓ)

Feed Medium (Concentration in Feed - 2.2ℓ Batch)

Glucose	1120	g (+/- 10 g)
Yeast extract	400	g (+/- 5 g)
Magnesium sulfate	34	g (+/- 2 g)

Supplemental Nutrients

Magnesium sulfate	1.0	g/ℓ (+/- 0.5 g/ℓ)
Leucine	1.0	g/ℓ (+/- 0.5 g/ℓ)
Vitamins	1.0	ml/ℓ (+/- 0.5 ml/ℓ)
Trace elements	1.0	ml/ℓ (+/- 0.5 ml/ℓ)

EXAMPLE 2. Process Development

To determine the different effects of shaker flask incubation, batch fermentation, and fed-batch fermentation on plasmid production by microorganism cultures, these three culturing methods were compared. The *E. coli* of Example 1 were grown in all three kinds of culture conditions. For both the shaker flask method and the batch fermentation, a Batch Medium of Terrific Broth, as shown below, was used. In the fed-batch fermentation, Fed-Batch Base Medium, also shown below, was used.

The shaker flask culture was maintained at 30 °C with shaking at 300 RPM, and the batch fermentation was kept at 30 °C with shaking at 600 RPM. The fed-batch fermentation process proceeded essentially as described in Example 1, except that the Feed Medium of Example 2 contained L-leucine, based on the result of the experiments of Example 1. Under the optimized scale-up conditions of the fed-batch fermentation, plasmid yield was dramatically increased relative to the corresponding culture incubated on the shaker or in the batch fermentation.

Batch Medium (Terrific Broth)

Yeast extract	24 g/ℓ (+/- 2 g/ℓ)
Tryptone	12 g/ℓ (+/- 2 g/ℓ)
Potassium phosphate, dibasic	2.3 g/ℓ (+/- 1 g/ℓ)
Potassium phosphate, monobasic	12.5 g/ℓ (+/-3 g/ℓ)
Glycerol	4 ml/ℓ (+/- 1 ml/ℓ)
Antifoam	0.25 ml/ℓ (+/- 0.1 ml/ℓ)
Conditions:	600 RPM, 30 °C

Fed-Batch Base Medium

Glucose	1.0 g/ℓ (+/- 1 g/ℓ)
Ammonium sulfate	5.0 g/ℓ (+/- 2 g/ℓ)
Yeast extract	5.0 g/ℓ (+/- 2 g/ℓ)
Potassium phosphate, dibasic	7.0 g/ℓ (+/- 2 g/ℓ)
Potassium phosphate,monobasic	8.0 g/ℓ (+/- 2 g/ℓ)
Magnesium sulfate	1.0 g/ℓ (+/- 1 g/ℓ)
Trace elements	1.0 ml/ℓ (+/- 1 ml/ℓ)
Vitamins	1.0 ml/ℓ (+/- 1 ml/ℓ)
Kanamycin	50 mg/ℓ (+/- 10 mg/ℓ)

Stock Trace Element Solution

$FeSO_4$ $7H_2O$	10 g/ℓ (+/- 1 g/ℓ)
$MnSO_4$ nH_2O	6 g/ℓ (+/- 0.6 g/ℓ)
$CoCl_2$ $6H_2O$	4 g/ℓ (+/- 0.4 g/ℓ)
$ZnSO_4$ $7H_2O$	2 g/ℓ (+/- 0.2 g/ℓ)
Na_2MoO_4 $2H_2O$	2 g/ℓ (+/- 0.2 g/ℓ)
$CuCl_2$ $2H_2O$	1 g/ℓ (+/- 0.1 g/ℓ)
H_3BO_4	0.5 g/ℓ (+/- 0.05 g/ℓ)
Concentrated HCl	13 ml/ℓ (+/- 1.3 ml/ℓ)

Vitamin Stock Solution

Riboflavin	0.42 g/ℓ (+/- 0.042 g/ℓ)
Pantothenic acid	5.4 g/ℓ (+/- 0.54 g/ℓ)
Pyridoxine	1.4 g/ℓ (+/- 0.14 g/ℓ)
Biotin	60 mg/ℓ (+/- 6 mg/ℓ)
Folic acid	40 mg/ℓ (+/- 4 mg/ℓ)
Thiamine	1.0 g/ℓ (+/- 0.1 g/ℓ)

Feed Medium (Concentration in Feed - 2.2ℓ Batch)

Glucose	1120	g (+/- 10 g)
Yeast extract	400	g (+/- 5 g)
Magnesium sulfate	34	g (+/- 2 g)
L-Leucine	25	g (+/- 2 g)

Conditions: 150-1500 RPM to control dissolved oxygen at 20%; 30 °C; pH 7.0 with ammonium hydroxide. Table 2, above, demonstrates the yield increases obtained when various constructs are grown under fed-batch conditions.

EXAMPLE 3. Identification of Optimum Specific Growth Rate for Plasmid Production

To identify the cellular specific growth rate for the optimal intracellular accumulation of plasmids, the *E. coli* culture of Examples 1 and 2 was grown under fed-batch conditions with varying rates of delivery of glucose. The variation in carbon delivery rates resulted in different cellular specific growth rates (μ values, as in the formula above).

A plot of plasmid DNA production (mg plasmid/g biomass) versus the various μ values demonstrates that plasmid DNA accumulation has an inverse association with growth rate, and that cultures with growth rates below 0.35/hour produce appreciably higher proportional yields of plasmid DNA than cultures with growth rates above 0.50/hour (Figure 1).

CONCLUSIONS

By applying the fed-batch fermentation principles to develop a fermentation manufacturing process for the production of recombinant plasmid DNA for clinical use, plasmid DNA yields were increased to milligram and gram quantities. This novel application for plasmid DNA manufacture reduces production costs by increasing overall processing efficiency. Identification of an optimum specific growth rate and application of a feeding strategy by a simple mathematical model allowed for the maintenance of high plasmid DNA per unit of biomass throughout a high-cell density fermentation.

Plasmid concentrations per unit of biomass were maintained during the increase in biomass under fed-batch fermentation conditions. In contrast, this ratio could not be maintained under batch fermentation conditions. Application of the fed-batch fermentation process to increase overall plasmid DNA yields is practical in a pharmaceutical manufacturing environment because yields are increased without the addition of chemicals such as chloramphenicol and without the necessity of altering the genotype of the host organism as in the case of relA mutants.

Although this invention has been described in the context of certain non-limiting preferred embodiments and examples, many alternative embodiments will be readily apparent to those of skill in the art. Accordingly, it is intended that the present invention be accorded the full lawful scope of the claims that follow, and lawful equivalents thereof.

WHAT IS CLAIMED IS:

1. A method for producing plasmid DNA in a microorganism, comprising the steps of:

providing a microorganism culture, wherein said microorganisms include a recombinant plasmid;

growing said microorganisms in culture under conditions that limit growth of said microorganisms during exponential phase to a growth rate of no more than about 0.35/hr; and then

purifying said plasmid DNA from said microorganisms.

2. The method of Claim 1, wherein said growing step further includes the step of measuring said growth rate of said microorganisms.

3. The method of Claim 1, wherein said growth rate is maintained by feeding one or more nutrients to microorganisms in quantities increasing at a rate approximately the same as the growth rate.

4. The method of Claim 3, wherein the actual growth rate of said microorganisms is monitored and the rate of increase in nutrient feeding is adjusted in response to said actual growth rate.

5. The method of Claim 3, wherein said feeding is done periodically.

6. The method of Claim 3, wherein said feeding is done continuously.

7. The method of Claim 1, wherein said conditions that limit growth are provided by controlled feeding of a carbon source to said culture.

8. The method of Claim 7, wherein said carbon source is selected from the group consisting of glucose, glycerol, sucrose, maltose, fructose, lactose, corn syrup, molasses, and honey.

9. The method of Claim 1, wherein said conditions that limit growth are provided by controlled feeding of an organic molecule for which said culture is auxotrophic.

10. The method of Claim 9, wherein said organic molecule is an amino acid.

11. The method of Claim 10, wherein said amino acid is Leucine.

12. The method of Claim 1, wherein said conditions that limit growth are provided by controlled feeding of an inorganic substance required by said microorganism for optimal growth.

13. The method of Claim 1, wherein said growing step further includes defining the appropriate medium to sustain optimal growth and plasmid production.

14. The method of Claim 1, wherein said growing step further includes optimization of culture parameters such as pH, temperature, and dissolved oxygen.

15. The method of Claim 1, wherein said microorganisms are prokaryotic cells.

16. The method of Claim 1, wherein said microorganisms are *E. coli*.

17. The method of Claim 1, wherein said microorganisms are eukaryotic.

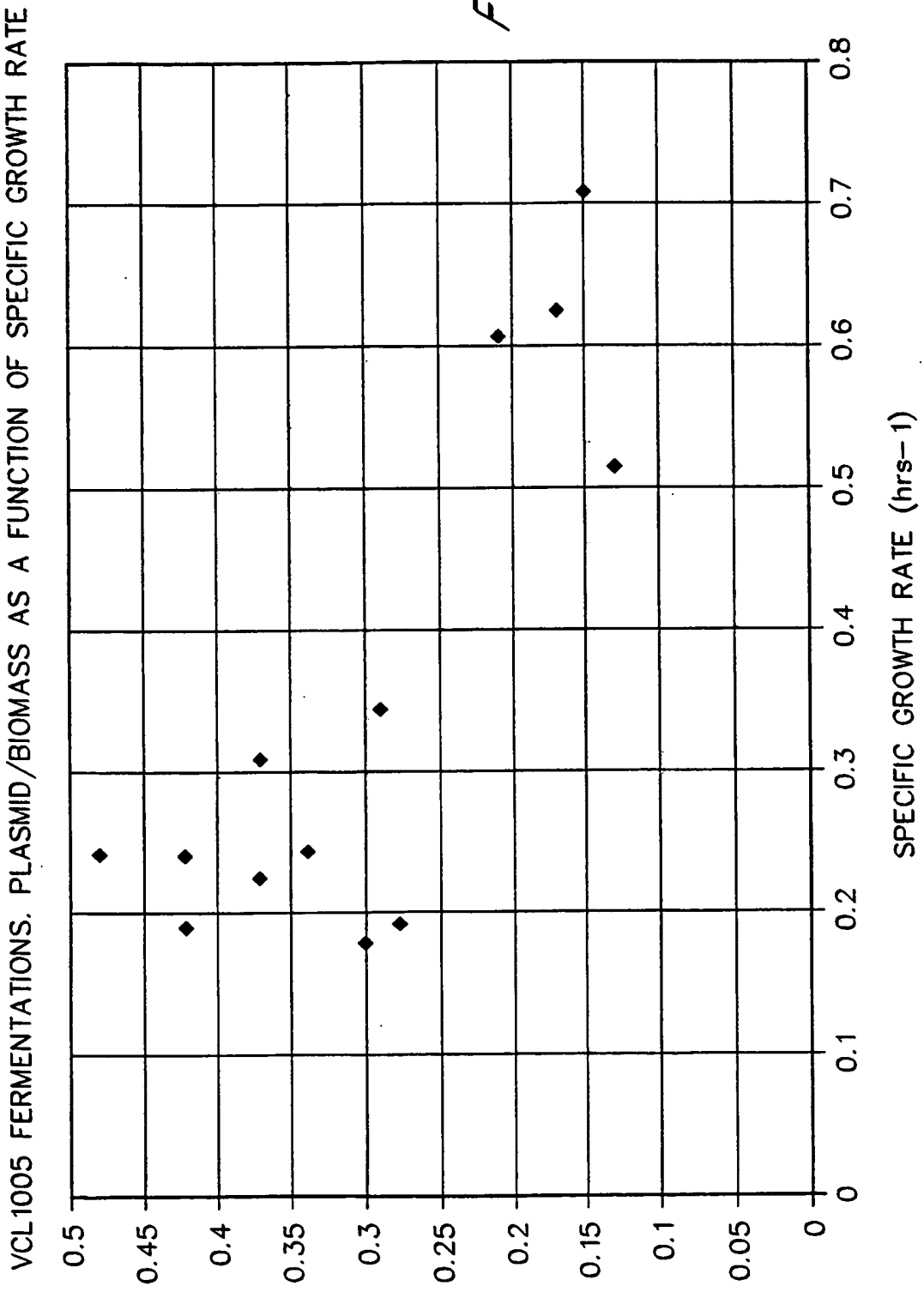
18. The method of Claim 1, wherein said microorganisms are yeast.

19. The method of Claim 1, wherein said purifying step results in pharmaceutical grade DNA.

20. The method of Claim 1, wherein the quantity of DNA produced by said purifying step is at least about 160 mg per 10ℓ batch.

21. The method of Claim 1, wherein the yield efficiency of plasmid DNA is at least about 0.04%.

FIG. 1


VCL1005 FERMENTATIONS. PLASMID/BIOMASS AS A FUNCTION OF SPECIFIC GROWTH RATE
PLASMID/BIOMASS (mg/g DCW)
SPECIFIC GROWTH RATE (hrs−1)
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
0.5
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8

INTERNATIONAL SEARCH REPORT

Interr ·al Application No
PC1/US 96/09746

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/10 C12P19/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DD,A,239 222 (VEB ARZNEIMITTLEWERK DRESDEN) 1986 see the whole document ---	1-21
Y	DD,A,299 380 (AKADEMIE DER WISSENSCHAFTEN) 1992 see page 3, paragraph 12 - page 4, paragraph 3 see page 6, paragraph 3 --- -/--	1-21

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
30 August 1996	06.09.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gurdjian, D

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INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/US 96/09746

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. BIOTECHNOL. (1994), 32(3), 249-59 CODEN: JBITD4;ISSN: 0168-1656, XP002012164 MENDOZA-VEGA, O. ET AL: "Production of recombinant hirudin by high cell density fed - batch cultivations of a Saccharomyces cerevisiae strain: physiological considerations during the bioprocess design" see examples 3,4	1-21
A	J. BIOTECHNOL. (1994), 32(3), 289-98 CODEN: JBITD4;ISSN: 0168-1656, XP002012165 HELLMUTH, K. ET AL: "Effect of growth rate on stability and gene expression of recombinant plasmids during continuous and high cell density cultivation of Escherichia coli TG1" see the whole document	1-21

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INTERNATIONAL SEARCH REPORT

. .ormation on patent family members

Interna sl Application No
PCT/US 96/09746

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DD-A-239222		NONE	
DD-A-299380		NONE	